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SUGGESTED PROCEDURES FOR INTERNATIONAL IDENTIFICATION OF LEGUME VIRUSES

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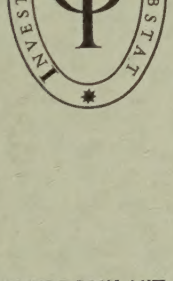

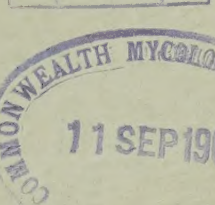

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SUGGESTED PROCEDURES FOR INTERNATIONAL IDENTIFICATION OF LEGUME VIRUSES

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SUGGESTED PROCEDURES FOR INTERNATIONAL IDENTIFICATION OF LEGUME VIRUSES[1]

Mit einer Zusammenfassung: Vorgeschlagene Verfahren zur internationalen Identifikation von Leguminosenviren

Met een samenvatting: Voorgestelde methoden voor internationale identificatie van leguminosenvirussen

BY

L. Bos[2], D. J. Hagedorn[3] and L. Quantz[4]

Introduction

Virus diseases of legumes have a world-wide distribution and are of great importance in agriculture. Therefore, they are being studied in several widely-scattered research laboratories. Since much confusion exists as to their exact identity and names, a proper diagnosis of these diseases is a major problem. Thus, need is felt for more international co-operation among researchers investigating these diseases. The aims are to make an international inventory of viruses attacking legumes by isolating, characterizing, and classifying them.

For these purposes standardized procedures for studying and describing viruses are essential to reduce confusion in the literature. Therefore, the procedures outlined below are being suggested for the identification (characterization and diagnosis) of legume viruses. Many of the procedures, however, should also apply to viruses in general.

The techniques of virus transmission by means of sap inoculation, grafting and dodder species, need no further description here. For investigations on transmission via soil and/or seed no special procedure has been described. Some suggestions for insect transmission will be given here, because results obtained depend largely on the way the experiments are carried out.

There are many problems in connection with identification of viruses found in legumes in the field. The first is to isolate the virus components from a probable "virus complex" because multiple infection in legume virus diseases is not uncommon. Then detailed studies must be made to describe the isolated components.

Of importance, especially from an agricultural point of view, is the study of host range, symptomatology, influence of environment, varietal reaction and methods of transmission. Virus identification based on host plant reaction, however, cannot always be considered conclusive since symptoms often depend, among other factors, on the species or variety used and on the environmental conditions. This often makes the so called "clinical" diagnosis unreliable. Consequently, it is rarely possible to get information from some publications, espe-

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cially the older ones, on the exact identity of the viruses concerned. Greater emphasis should be laid on the study of the intrinsic characters of the viruses by physicochemical tests and especially electron microscopy and serology. They form the basis for the only so called "etiological" diagnosis. Thus far the study of these characters has been restricted to viruses which are sap transmissible in one way or another.

Insect transmission studies have a practical meaning because they provide information which may lead to eventual control of the virus disease concerned. The study of virus-vector relationships can also help to characterize the virus. Strain relationships can be estimated by means of cross-protection tests. For those viruses which are not sap transmissible, host plant reaction, insect transmission and in some cases cross protection are the only available tools for diagnosis. Fortunately, an increasing number of viruses previously considered to be only insect borne appear to be transmissible in other ways, e.g. from plant to insect vector or from insect to insect (cf. BLACK, 1955). In the extracts of plants or insects used in this way, properties of the viruses can be estimated, even by means of electron microscopy and serology.

For some surveys and bibliographies on legume viruses we may refer to the well-known general textbooks on plant viruses and to HAGEDORN & WALKER (1954), KREITLOW e.a. (1957), WEISS (1945) and ZAUMEYER & THOMAS (1957).

SEPARATION OF THE VIRUS COMPLEX FOUND IN THE FIELD

The first problem with identification of viruses found in field-grown legumes is to know whether or not the infection is of complex nature and especially to separate out any strains of cucumber mosaic virus, of alfalfa mosaic virus and/or of some tobacco viruses which can infect legumes. Some suggested differential hosts for this initial broad separation are:

Bean – *Phaseolus vulgaris* L. (e.g. variety Bountiful or Beka),
Broad bean – *Vicia faba* L.,
Pea – *Pisum sativum* L. (e.g. variety Perfected Wales, W. R. Perfection, Eroica, or Mansholt's Pluk),
Red clover – *Trifolium pratense* L.,
Cucumber – *Cucumis sativus* L. (National Pickling),
Tobacco – *Nicotiana tabacum* L. (Samsun or White Burley).

In performing this initial test also an orientation concerning the identity of the virus will be obtained. This will help in selecting an appropriate range of host plants from among the list given below. This test will provide more accurate information concerning the possible presence of virus components or strains. In some cases (e.g. to distinguish between the bean viruses 1 and 2) a quick method like the "Schalentest" (QUANTZ, 1957) may be useful.

In isolating components from a complex it might be useful to employ local lesion hosts when possible. Physical property tests are also useful sometimes, as are insect transmissibility trials. If a preparative ultracentrifuge is available, separation of components may also be achieved by means of density gradient rate or equilibrium ultracentrifugation (BRAKKE, 1953). Electrophoretic separation is also possible (BRAKKE, 1955).

Host plant reaction

Study of host plant reaction is one tool in virus identification. For those viruses which are not sap transmissible it is an essential, and, together with the study of insect vectors, one of the few available tools in diagnosis. It should be stressed that when a mixture is involved the host plant reaction, both regarding symptomatology and resistance, may be different.

Host range

Most viruses attack specific host plants. It would be very desirable to adopt, on an international basis, a recommended list of hosts (including genus, species, and variety). These species should be made available to all interested researchers. In this way investigators throughout the world would be using the same hosts and results would be more meaningful. Some suggested hosts are listed below.

Scientific Name	*Common Name*	*Variety*
Arachis hypogaea L.	Peanut	
Cicer arietinum L.	Chick Pea	
Cassia tora L.	Sickle Pod	
Crotalaria spectabilis ROTH.	Showy Crotalaria	
Dolichos lablab L.	Hyacinth Bean	
Glycine soja SIEB. & ZUCC.	Soy Bean	Blackhawk
Lespedeza striata (THUNB.) H. & K.	Common Lespedeza	
Lupinus albus L.	White Lupine	
Medicago sativa L.	Alfalfa (Lucerne)	
Melilotus alba DESR.	White Sweet Clover	
Phaseolus lunatus L.	Lima Bean	
Phaseolus vulgaris L.	Bean	Beka
		Bountiful
		Pinto
		Saxa or Genfer Markt
		Topcrop
Pisum sativum L.	Pea	Foli
		Juwel
		Mansholt's Pluk
		Perfected Wales
		Wisconsin Perfection
Trifolium hybridum L.	Alsike Clover	
Trifolium incarnatum L.	Crimson Clover	
Trifolium pratense L.	Red Clover	Median
Trifolium repens L.	White Clover	White Dutch
Vicia faba L. forma *minor*	Broad Bean	
Vicia sativa L.	Spring Vetch	
Vicia villosa ROTH.	Hairy Vetch	
Vigna sinensis (L.) ENDL.	Cowpea	Black
Chenopodium amaranticolor COSTE & REYN.		
Cucumis sativus L.	Cucumber	National Pickling
Capsicum annuum L.	Pepper	
Datura stramonium L.	Jimsonweed	
Gomphrena globosa L.	Common Globe Amaranth	
Nicotiana glutinosa L.	Clammy-leav'd Tobacco	
Nicotiana rustica L.	Aztec Tobacco	
Nicotiana tabacum L.	Turkish Tobacco	Samsun
	Burley Tobacco	Kentucky 57
Zinnia elegans JACQ.	Zinnia	

For the special benefit of researchers in warm climates it has been suggested that other tropical legumes be included such as *Cajanus indicus* SPRENG. (pigeon pea), *Canavalia ensiformis* DC. (jack bean), *Centrosema pubescens* BENTH. (butterfly pea), *Crotalaria juncea* L. (sunn hemp), and *Stizolobium deeringianum* BORT. (velvet bean).

It would be desirable to use only clones of the clovers listed because of the heterozygosity among plants within a variety, but this might not be feasible due to quarantine regulations.

Whether or not symptoms are observed after inoculation of these plants, back inoculation to a known susceptible host is necessary to make results scientifically acceptable. By means of back inoculation it is also possible to distinguish between systemic and local infections. Infection also can be checked by means of electron microscopy and serology.

Symptomatology

It would be extremely useful if a uniform system of describing symptom expressions could be adopted by all those working with legume viruses. Perhaps, definitions for terms used eventually could be devised so that everyone would know more exactly what was meant by the descriptions given. A helpful paper in this regard entitled "Symptoms of Virus Diseases in Plants" will be published soon by BOS. Symptom studies should be made at as nearly normal greenhouse temperatures as can be obtained (20–22 °C). If departure from this temperature is inevitable, researchers should so indicate. Normal illumination should also be furnished and data submitted to indicate day length and approximate number of foot candles of light available to the growing plant.

Data may be gathered on incubation period and close observation of plants should then result in the recording at the appropriate time of (1) initial symptoms, (2) "secondary" symptoms, and finally (3) typical symptoms if different from (1) or (2). Local and systemic symptoms and the over-all effect on the plant may also be noted. Notable symptoms on all plant parts should be recorded.

When symptom information is given in tabular form and abbreviations are used the following guide may be useful.

Chl = Chlorosis	Ma = Malformation	TN = Top necrosis
En = Enations	Mo = Mottle or Mosaic	VB = Vein banding
FB = Flower breaking	N = Necrosis	VC = Vein clearing
LC = Leaf curl	RS = Ring spot	WB = Witches' broom
LL = Local lesions	Str = Streak	W = Wilting
LR = Leaf roll	Stu = Stunt	Lat = Latent

Influence of environmental conditions

It is often helpful to know effects of environment, especially temperature, upon disease development and symptom expression. This holds true for studies with legume viruses as well as other plant pathogens. In studying temperature effects, plants to be tested should be grown initially at normal greenhouse temperatures (20–22 °C), inoculated at the proper stage of development[1] and then equal numbers, and 4 to 6 replicates, should if possible be placed at specific temperatures. Such temperatures as 16, 20, 24 and 28 °C may be maintained in temperature

[1] The leaves should be fully expanded but still young. It's impossible, however, to give generally acceptable directions for all viruses and all host species.

chambers of limited size or even in greenhouses. Data are then recorded in a manner similar to that described under *symptomatology*. Particular attention should be paid to incubation period and final over-all effect on the host plant.

Varietal reaction

From an agricultural point of view it is very important to know the crop varietal reaction to the viruses studied. This in certain instances can also be useful to characterize the viruses and especially virus strains. The comparison of pea and bean varietal reaction is especially useful with pea and/or bean virus strains. Such an investigation entails (1) the growing of a selected number of representative varieties of these crop plants under similar conditions, (2) inoculating them with the same inoculum at the same time, and then, (3) after a proper incubation and note-taking period, making back inoculations to a known susceptible host.

Some suggested pea and bean varieties are listed below

Peas	*Beans*
Alaska	Beka
Alderman	Bountiful
Edelperle	Genfer Markt
Eroica	Idaho Refugee
Foli	Imuna
Horal	Kentucky Wonder
Juwel	Michelite
Mansholt's Pluk	Prince
New Era	Red Kidney
Perfected Wales	Stringless Blue Lake (White seeded)
Thomas Laxton	Stringless Green Refugee
W.R. (Wis.) Perfection	Topcrop

Notes on varietal reaction may be simple or involved. In some cases enough information may be supplied if the data merely indicate whether or not a particular variety is resistant or susceptible. In other cases, for instance where strains of bean or pea viruses are being investigated, it would be helpful to make more detailed notes on how the varieties react so far as symptoms are concerned. Especially in the bean varieties it is important to note whether there is a general immunity or the virus causes a local or a systemic infection.

Physicochemical properties

Regarding the biochemical nature of the incitants of plant virus diseases, the physico- or biochemical property studies form an essential tool for the description of intrinsic properties of viruses.

There are two groups of physicochemical properties, viz.: those studied in *plant sap* and those studied in more or less *purified suspensions*. Both groups have to be distinguished.

The first group of properties is commonly known, and usually exists of tolerance to dilution, thermal inactivation and aging in vitro. They were considered to be of importance for description and classification of plant viruses by Johnson (1927) and have been described in some detail by Johnson & Grant (1932). These properties, however, are studied in sap of plants. The source plants may be different taxonomic entities, and may have been grown under different con-

ditions with the result that the virus titre is quite variable. Moreover, the properties are assayed in biological infectivity tests. Therefore the results obtained oftentimes vary considerably and thus are *rather qualitative*. Since they are often used as to their easy application, however, and since under the above restrictions they may give valuable information, the procedures will be shortly described.

In contrast to the first group of properties, in which more or less the variable host reaction is involved, the second group of properties represents more fundamental characters of the viruses themselves. Therefore, the estimation of properties such as sedimentation constant, ultraviolet absorption and other more chemical characters, in addition to serology and electron microscopy, are of great importance. So far, they cannot be easily applied for practical purposes, and in many virus laboratories facilities are still lacking. Improvement of facilities, equipment concerned, and proper interpretation of results has to be stressed.

Physicochemical properties in plant sap

To standardize procedures in using crude plant sap, juice should be taken from relatively young plants showing typical symptoms. It is important to record the environmental conditions existing during the course of the studies and to give the species, variety, age, size, etc., of the plants used for virus source and for assay. To stabilize pH and ionic activity in the virus containing plant sap, it is advisable to dilute the original plant sap 1 : 10 with 0.01 M phosphate buffer pH 7. The juice need not be pretreated in other ways, e.g., no chloroplasts removed.

It is advisable to assay the presence of virus by means of local lesion hosts in order to obtain more constant results. This is due to the fact that, e.g., with aging in vitro tests and thermal inactivation tests virus inactivation takes place more or less according to a logarithmic curve. Only very few surviving particles may fail to incite local lesions, whereas they may produce a systemic reaction. Due to the more or less asymptotic course of the logarithmic line this end point is not clear-cut but rather variable. For instance, alfalfa mosaic virus has a thermal inactivation point, according to the textbook of KENNETH SMITH, between 62 and 70 °C. From the results obtained by MILBRATH (1959) with 24 strains of this virus it is evident that the end point obtained by means of cowpea as a local lesion host is about 46 °C.

In using a local lesion host, however, the end point still depends upon the original concentration of virus particles, the sensitivity of the assay plant, and the rate of inactivation. Since the first two factors are variable and usually unknown for a given sample and a given test plant under given circumstances, YARWOOD & SYLVESTER (1959) recently suggested that the rate of inactivation in aging in vitro tests be determined by computing the half-life of the virus according to the equation:

$$t_{\frac{1}{2}} = \frac{T \log 2}{\log P_0 - \log P_1}.$$

In this equation P_0 = original population, P_1 = population after time T, T = interval of observation, and $t_{\frac{1}{2}}$ = time interval for half of the individuals to be inactivated, or the half-life of the population.

Instead of taking P_0, P_1, and T from the observations, it is suggested (BANCROFT, personal communication) that the logarithms of the numbers of local lesions be plotted against the time. Then one should adjust the resulting straight line statistically according to the method of least squares. The P_0, P_1, and T values are then derived from this line. In case the virus has a very long longevity one can already estimate the half-life from a part of the observations.

In indicating the end point in these physicochemical tests two critical points should be recorded. These are (1) the last point where virus activity can still be

'observed and (2) the first point where virus activity can no longer be demonstrated.

Tolerance to dilution

There are several recognized methods for preparing dilutions of plant viruses, one of which will be outlined here. Extracts from plants obtained according to the methods described above are placed in a test tube. Distilled water is put in other test tubes, 9 ml per tube. A 1 ml volumetric pipette is used to transfer 1 ml of agitated infective sap to the first of the test tubes containing 9 ml of distilled water. This solution is thoroughly mixed and another pipette is used to transfer 1 ml of this solution to the next tube of distilled water. This procedure is repeated until dilutions of 0, 1:10, 1:100, etc., up to 1:10,000,000 have been made. Inoculations with these dilutions are made to a susceptible host (if possible a local lesion host), a proper incubation period is given, and readings are made when typical symptoms appear on the control (0 dilution). More meaningful results will be obtained if the same number of plants are inoculated with each dilution. Since at higher dilutions the incubation period may be lengthened, the test plants concerned should be under observation for a longer period.

Thermal inactivation

Virus-containing plant extracts are placed in a test tube to serve as the virus reservoir. Thin-walled glass tubes are then used for holding the virus in the agitated hot water bath. The tubes often used in serology are very suitable. They have a thin wall of about 0.7 mm, an outside diameter of 10 mm, a length of 10 cm and a total capacity of about 5 ml. These tubes are filled with about one or two ml of virus-containing sap. This must be done carefully so that no plant extract is on the upper wall of the tube and therefore not immersed in the hot water. Then the tubes are placed between clips underneath the cover of the water bath. This cover is then carefully put on top of the bath with a proper temperature of the water. In doing so, the tubes are immersed in the water with a level adjusted previously to at least 3 cm above the level of the plant extract within the tubes. The technical layout may differ, but it is essential to use narrow thin-walled glass tubes.

The water bath should be well insulated so that water temperature can be accurately regulated and maintained. It is important to have adequate agitation of the water within the bath.

The tubes should be heated for 10 minutes after immersion at temperatures from 50–95 °C using intervals of 5 °C. When, from preliminary host range tests and symptomatology, some information is already known concerning the identity of the virus, the range of temperatures to be tested may often be lessened. In this case and/or after having obtained some rough information about the inactivation point in a test covering the whole range with intervals of 5 °C, a second test may be performed to estimate more exactly the thermal inactivation point by using a number of intervals of 2 °C within the range in which the exact temperature lies. However, there is no widespread agreement as to how meaningful the more precise test will be.

Immediately after the 10-minute heat treatment the tubes and contents should be quickly immersed in very cold water (flowing if possible) where they should remain until thoroughly cooled. The viruses are then rubbed into susceptible

plants (preferably local lesion hosts) of uniform age, etc., and grown under like conditions. Readings are made when typical symptoms develop on the control plants. One should again be aware of the fact that with the lower virus concentration, e.g., due to treatment at a higher temperature, the incubation period may be lengthened. This may be especially true for hosts expected to develop systemic symptoms.

Aging in vitro

The aging *in vitro* studies consist simply of storing virus containing plant extracts at room temperature (20–22 °C) in stoppered test tubes or small bottles and testing for virus infectivity after progressively longer storage periods. A geometric progression of aging times in days might go something like this: 1, 2, 4, 8, 16, 32 and 64. Perhaps, to subject a given quantity of virus to the same conditions, it would be well to store all the virus extract in the same container, and then at the appropriate times remove a certain aliquot to test infectivity by inoculating proper host plants. Again, a sufficient incubation period is given to allow symptom development; then adequate notes are taken on the ability of the virus to withstand aging. The addition of chemicals to prevent fungi and bacteria from developing should be avoided since their influence on the virus concerned is many times unknown. If they are used, proper controls to evaluate their effects must be included. When possible, researchers should avoid making comparisons with viruses tested from different host plants. Instead, in the interest of standardization, it is suggested the Perfected Wales peas be used as source plants for virus extracts to be aged.

Physicochemical properties in purified suspensions

Our knowledge of the behavior of viruses *in vitro*, and even in more or less purified suspensions outside of the plant sap, is rapidly increasing. Such investigations provide us with very helpful information for characterizing the viruses themselves. So far, however, the experiences in purifying viruses are too few to outline standardized general procedures. In view of the importance of data obtained in this way for an exact description and classification of viruses, improvement of methods of virus purification and of studying the physicochemical properties *in vitro*, cannot be emphasized enough.

A few characters which can be studied rather easily, when some simple laboratory tools are available, will be mentioned here.

An important technique for distinguishing different viruses is the rate of sedimentation through a density gradient column, according to BRAKKE (1958). This rate depends upon size, shape, and density of the virus concerned, and appears to be constant for most viruses. With the help of a simple preparative ultracentrifuge, this rate can be easily measured at low concentrations of virus in comparison to some standard like tobacco mosaic virus. For technical details we refer to the above mentioned publication.

If available, an electrophoresis apparatus may provide valuable information concerning the electric charge of virus particles and may help in estimating the iso-electric point. If no such apparatus is at hand, study of the behavior of the virus in partially purified preparations at different pH levels may give useful information.

By means of a spectrophotometer the virus protein content of a given suspen-

sion can be estimated, whereas combined phosphorus analysis may provide information as to the nucleic acid content of the virus under investigation.

Electron microscopy

Evidence obtained in virus research thus far suggests that morphology and size of virus particles are quite constant and are characteristic features of viruses. This makes electron microscopy an important tool in identifying viruses. As yet only a few legume viruses have been studied in this respect.

The virus containing plant sap can be applied on to the grids covered with a thin layer of collodion or formvar in several ways. Until now no general valuable method has been developed. Three methods will be mentioned here:

the *exudate method* (Johnson, 1948),
the *spray method* (Gold, Scott & McKinney, 1957) and
the *dip method* (Brandes, 1957).

The exudate method is still valuable but it is advisable to use a single leaf instead of a whole plant. After the drop of sap has been put on the grid it should be flattened out by blowing thereon or by using a needle (Brandes, personal communication).

The two latter methods were used and discussed by Hagedorn, Bos & van der Want (1959). Especially the dip method appears to have many advantages, such as to be very easy and to induce very little breakage of particles. No results with "spherical" particles have been obtained with any of these three methods. Presumably some purification is necessary for such viruses since plant sap contains normal protein particles which appear to be spherical.

In all these methods the grids covered with scattered virus particles can be sprayed with a suspension of about 0.01 % solids of polystyrene latex balls with a calibrated diameter of e.g. 340 mμ to serve as a standard for the magnification of the electron microscope. This is especially important for devices with electromagnetical lenses having fluent magnification in contrast with electrostatic lenses. The value of this standard is not generally accepted, however, (cf. Brandes & Paul, 1957). Instead of polystyrene latex balls it might be useful to use a virus with known length, general availability, which is easily prepared, e.g. tobacco mosaic virus. This virus could be added to the preparation concerned or photographed separately in the same series with the virus to be measured.

If in a certain laboratory no electron microscope is available, the preparations can easily be made and sent to a center where they can be shadowcast and studied by means of the electron microscope.

Particle size measurements can be made in several ways. A good technique is to project the picture (e.g. magnification × 2000) onto a screen or white wall. There the particles can be measured in mμ using flexible rulers designed for the specific magnification thus obtained. The results can be scored immediately on a mimeographed paper (Fig. 1) based on particle size frequencies of 25 mμ (e.g. 400, 425, 450, 475 mμ etc.). By this procedure a frequency curve develops at the time of measurement.

A detailed and critical description of the methods in virus measurements and a comparison of virus lengths is given by Brandes & Paul (1957). If the electron micrographs and measurements are made accurately enough, standard lengths

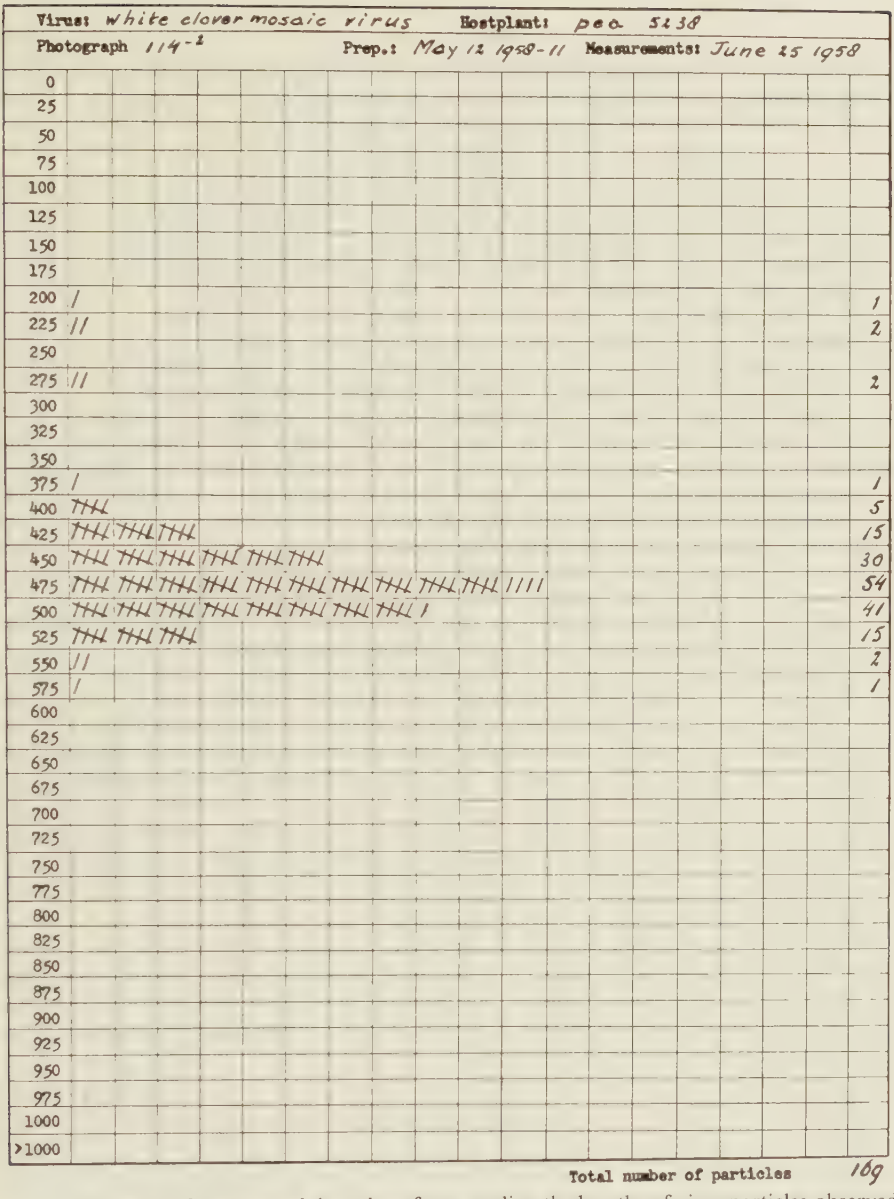

FIG. 1. Example of a suggested data sheet for recording the lengths of virus particles observed in electron micrographs. The numbers in the vertical column on the left indicate virus particle lengths in millimicrons. As particles are measured, marks are made in the appropriate spaces, and finally, totals are given in the right column.

Muster einer Liste für die Auswertung der Längenmessung an Viruspartikeln nach elektronenmikroskopischen Aufnahmen. Die Ziffern in der ersten Spalte geben die Partikellängen in mμ an. Bei der Messung der Teilchen werden die Längen in den jeweiligen Querreihen angestrichen und abschliessend in der rechten Spalte addiert.

Voorbeeld van een formulier ter weergave van de lengten van de virusdeeltjes, waargenomen in elektronenmicroscopische opnamen. De getallen in de linker kolom geven de lengten der virusdeeltjes aan in millimicrons. Tijdens het meten der deeltjes worden de lengten in de betreffende horizontale kolommen aangestreept, terwijl na afloop de totale aantallen in de rechter verticale kolom worden vermeld.

of viruses can be calculated by means of the statistical methods described in their publication.

Serology

Besides electron microscopy, serology plays an essential part in identifying viruses. For a number of legume viruses antisera are already available. Exchange of antisera will be very important in studying relationships between virus isolates found in different countries.

Since in preparing antisera different methods do not lead to fundamental differences, as far as is known, these methods need no further description here.

As to differences in behavior of different viruses it is not possible to suggest generally acceptable methods of pretreating the virus-containing plant sap and of performing the serological reactions. In many cases absorption of the antiserum with sap of healthy plants to remove antibodies against normal constituents can be avoided. Dilutions both of serum and plant sap should be made preferably with buffered saline. Proper controls of sap of diseased and of healthy plants with normal serum and buffered saline must be included.

For routine purposes sometimes the *precipitin* and the *agglutination reaction*, both on slides, have been valuable. Especially for studying quantitative relationships between viruses the precipitin reaction is most commonly used, both in tubes incubated in a waterbath or according to the *microreaction under paraffin-oil* as developed by van Slogteren (1955b). The latter method is of great value due to its easily surveyable results, and its need of very small quantities of antiserum. The *precipitin-ring* test appears to be very critical and may sometimes be more sensitive than the microreaction method, but can only be used with rather pure virus preparations.

Although the *agar-gel-diffusion method* (van Slogteren, 1955a) has certain advantages, this method is not suitable for elongated, threadlike particles. Sometimes the complement-fixation method, and the nitrogen-determination method (cf. e.g. Matthews, 1957) may be used.

To make results obtained in different laboratories more comparable, a uniform way of recording is suggested, as is indicated in the adjacent table. Data should be read after a fixed time under controlled conditions. To differentiate the observations and record the speed of reaction, results also can be read after e.g. 5, 15, 30, 60 and 90 minutes.

Cross protection

Good evidence of virus interrelationship may sometimes be obtained through the use of cross-protection tests. Using the hypothesis that strains of one and the same virus will protect the host plant against each other, appropriate host plants are inoculated with one virus and after typical systemic symptoms develop a second virus is then mechanically introduced into the systemically-infected tissue. If the viruses are related generally no signs of the presence of the second virus are observed; if they are not related, evidence that the second virus is present will become apparent.

Obviously a host must be used which develops characteristic differential symptoms upon inoculation with the two viruses under study. Especially suit-

TABLE 1. Example of recording results of a serological test.
Reaction of the Dutch isolate RK 5 and a Wisconsin isolate of the red clover vein-mosaic virus with the antiserum prepared against the Dutch isolate.
Muster für die Wiedergabe eines serologischen Testes.
Reaktion des holländischen Isolats RK 5 und eines Isolats des Rotkleeadernmosaik-Virus aus Wisconsin mit einem Antiserum gegen das holländischen Isolat.
Voorbeeld van de weergave der resultaten van een serologische proef.
Reactie van de Nederlandse isolatie RK 5 en een isolatie van het nerfmozaïekvirus van rode klaver uit Wisconsin met het antiserum bereid tegen de Nederlandse isolatie.

	Dilutions of sap of peas *Verdünnungen des Saftes von Erbsen Verduningen van sap van erwten*	Antiserum				Normal serum	Saline *Phys. Kochsalzlösung Fysiologisch zout*	
		1/4	1/16	1/64	1/256			
Dutch isolate	1/4	+++[1]	++	++	–	–	–	–
Holländisches Isolat	1/16	++	++	+	–	–	–	–
Nederlandse isolatie	1/64	–[2]						–
Wisconsin isolate	1/4	+++	+++	++	+	–	–	–
Isolat aus Wisconsin	1/16	++	++	+		–	–	
Isolatie uit Wisconsin	1/64	–					–	–
Healthy pea	1/4	–			–		–	
Gesunde Erbse	1/16		–			–		
Gezonde erwt	1/64	–		–	–		–	–

[1] The number of + signs is an indication for the quantity of precipitate.
Die Anzahl + zeigt die relative Stärke des Präzipitats an.
Het aantal + tekens is een maat voor de hoeveelheid precipitaat.
[2] No precipitate.
Kein Präzipitat.
Geen precipitaat.

able for cross-protection tests are those strains inducing local necrotic lesions, a systemic necrosis or a bright mosaic on one plant species, variety or clone in contrast with the virus strains to be tested. The latter must produce a fully systemic reaction. As research progresses the number of hosts showing distinct symptoms with different strains of legume viruses and which thus may be used in cross-protection tests will increase. For example, newly discovered hosts for cross-protection tests with strains of bean virus 2 are *Crotalaria spectabilis* ROTH. (CORBETT, 1957) and red clover *(Trifolium pratense* L.) clone KyC 71-8 (DIACHUN & HENSON, 1958a, 1958b).

INSECT TRANSMISSION

For distinguishing the characteristic features of legume viruses it is also useful to determine whether the virus is insect transmissible and, if so, to identify the vector(s) and to determine the insect-virus relationship. It is essential to know the genus and species of the insects which are capable of transmitting a given virus. Likewise, it should be discovered whether the virus is persistent or non-persistent and the relative ease of transmission, as determined by the characteristics indicated below, should also be studied.

Acquisition time[1]*:* Period of time that a noninfective vector is fed on or has access to a virus source.

Acquisition threshold: Minimum time necessary for a vector to feed upon or to have access to a source in order to transmit the virus.

Inoculation time[1]*:* Time the vector feeds upon or has access to the healthy host.

Inoculation threshold: Minimum time necessary for a viruliferous insect to feed upon or to have access to a healthy plant in order to transmit the virus.

Latent period: Common term used to denote the time that elapses before a vector can be demonstrated to be infective after the acquisition threshold.

Aphid transmission tests should be made as soon as possible after the virus has been obtained from the field because continued mechanical transmission may definitely affect aphid transmissibility.

The experiments should be conducted by using aphids or leafhoppers which are believed to be the most effective vectors of the virus under investigation. Good vectors are in general *Myzus persicae* SULZ., *Acyrthosiphon pisum* HARRIS *(A. onobrychis* B.D.F.) and *Macrosiphum solanifolii* ASHM.

In the following paragraphs some suggested procedures for the use of ophids are outlined.

Determining acquisition threshold

Nonviruliferous mature aptera are starved for 4–8 hours and placed on virus-infected plants. One aphid per plant is used and acquisition feeding periods of 15 seconds (if possible), 1 minute, 5 minutes, and 1 hour, are timed with a watch. Timing is begun as soon as the aphid puts its proboscis in a permanent feeding position on the plant. By observing the proboscis under a 12 × hand lens, it can be determined when it becomes permanently stationary. Many times a few preliminary probes are made of about 10–15 seconds duration. One cannot be sure whether or not the insect has fed unless positive transmission results are obtained. For longer acquisition feeding periods, such as 4, 8 and 24 hours, 10–15 aphids per plant are placed on infected plants for these times, so the exact period is not known. This could be more clearly defined by using the first described technique.

After the acquisition time the aphids are moved to healthy test plants for the inoculation time of 24 hours after which they are killed with an aphicide.

Determining inoculation threshold

Aphids which have had an optimal acquisition time on infected plants are given inoculation times of 1, 5, 10 and 20 minutes and maybe even 1, 4, 8, 24 and 48 hours. Five aphids are placed on each healthy plant and the period is timed in a manner similar to that described above. After the test the aphids are killed.

Studying starvation effect

Aphids are starved for 4–8 hours and allowed to feed on diseased plants for 1 minute and 5 minutes, after which they are given an inoculation feeding period

[1] Since recent investigations indicate that nonpersistent viruses may be transmitted only after probing or puncturing by a vector, the often used terms "acquisition feeding" and "inoculation feeding" may only hold for persistent viruses.

of 24 hours on healthy plants before being destroyed. A similar experiment using unstarved aphids must be performed for comparison.

Results are obtained after sufficient time has elapsed for typical symptoms to develop.

Remember – in handling aphids, use a moistened, fine, camel's hair brush, and tease, don't force, the aphid loose from its feeding station.

Conclusion

Experimental results obtained anywhere by carefully using the procedures described above should be more easily comparable. Exchange of these results and especially of antisera and of results obtained by means of electron microscopy are highly recommended. On this basis an international inventory of legume viruses and a tentative grouping of them may be possible.

For these purposes a closer international co-operation between specialists working on the group of legume viruses is suggested. Such an international co-operation and exchange of material and results will be the only real basis for tackling problems concerning classification, nomenclature and identification of plant viruses in general.

Publications on supposed new viruses should be based on the procedures described here so as to present as much evidence as possible that the virus differs from those already described. In this way future added confusion in literature will be greatly reduced.

In old legume virus publications description was on a less scientific basis; so many times the relationship between the viruses under investigation more recently and those described earlier cannot be established accurately. Therefore the use of old names of viruses should be avoided unless good evidence is ob, tained that they are currently applicable.

It is important to preserve cultures of originally-described virus so that the are always available as type material. Preferably the virus material shouldbey preserved by cool-drying – finely-cut plant material placed in dessicators over anhydrous calcium chloride at about 4 °C and stored under the same conditions.

Summary

The importance and need for standardized procedures in the international identification (description and diagnosis) of legume viruses is pointed out and suggestions are given to aid in this accomplishment. Outlines of techniques for using common methods such as host range studies, symptomatology, physical properties tests, cross-protection tests and insect transmission data are presented. Likewise, suggestions are given for the use of some of the newer approaches including physicochemical property studies, serology, and electron microscopy. The importance of these latter studies for virus characterization is emphasized. Some proposals for the uniform recording of results have been put forward. Most of the procedures may also be useful for the identification of viruses in general.

Zusammenfassung

Die internationale Bedeutung der Untersuchungen über Viruskrankheiten an Leguminosen macht die Anwendung einheitlicher, vergleichbarer Methoden zur

Virusbeschreibung notwendig. Unter diesem Gesichtspunkt werden daher Methoden zur Untersuchung der Wirtspflanzenkreise, der Symptomatologie, der Insektenübertragung, der Prämunität sowie der chemo-physikalischen, serologischen und elektronenmikroskopischen Merkmale zusammengestellt. In der Literatur ist vielfach eine Unsicherheit über die Identität der Erreger von Viruskrankheiten anzutreffen; daher wird besonderer Nachdruck auf die letztgenannten Methoden gelegt, die wesentliche Eigenschaften der Viren selbst erfassen und nicht auf die veränderliche Reaktion der Wirtspflanzen zurückgehen. Diese Arbeitsmethoden, die zumeist auch allgemein für weitere Pflanzenviren Gültigkeit besitzen, bieten eine gute Basis für die vergleichende Virusbeschreibung und damit die Voraussetzung für eine allgemeine Virusklassifikation wie auch für die praktische Virusdiagnose. Für diese Arbeiten ist die internationale Zusammenarbeit durch Austausch von Antiseren, Saatgut usw. zu verstärken.

Eine Anzahl wertvoller Hinweise zu diesem Beitrag kamen von Fachkollegen aus allen Teilen der Welt, denen das Manuskript vorher übersandt worden war.

Samenvatting

Met het oog op de internationale betekenis van het onderzoek over virusziekten van vlinderbloemigen wordt gewezen op de grote waarde van en de behoefte aan gestandaardiseerde, internationaal aanvaarde en toegepaste methoden ter identificatie (karakterisering en herkenning) van de bij deze plantesoorten en gewassen voorkomende virussen.

In deze publikatie worden de technieken geschetst ter bepaling van de waardplantenreeks en ter bestudering van de symptomatiek, de fysische eigenschappen, premunitie en overdracht door insekten der onderhavige virussen. Tevens worden suggesties gegeven voor de toepassing van nieuwere methoden van onderzoek, zoals die ter bestudering van de fysisch-chemische eigenschappen, de serologie en de elektronenmicroscopie. Enige uniforme wijzen van vermelding van de resultaten van onderzoek worden voorgesteld.

Het manuscript van deze bijdrage is beoordeeld door collega's in verscheidene delen der wereld en op grond van ontvangen suggesties aangevuld met een aantal waardevolle opmerkingen.

Het merendeel der in het rapport beschreven werkwijzen wordt van waarde geacht voor de beschrijving van plantevirussen in het algemeen. In de virusliteratuur bestaat veel verwarring wat betreft de juiste identiteit der verwekkers van virusziekten. Daarom wordt in deze bijdrage vooral de nadruk gelegd op de laatstgenoemde aspecten van het virusonderzoek ter omschrijving van intrinsieke viruseigenschappen. Deze eigenschappen vormen de betrouwbaarste basis voor de karakterisering en beschrijving van virussen, daar ze betrekking hebben op de virussen zelf en niet op de veranderlijke reactie der waardplant. Meer fundamenteel onderzoek over deze methoden van virusbeschrijving wordt dan ook van wetenschappelijke betekenis geacht voor een algemene virusclassificatie en van grote praktische betekenis voor een daarop berustende etiologische diagnostiek.

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